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ANNEX 4

New Coupling Agents for the Synthesis of Immunotoxins Containing a Hindered Disulfide Bond with Improved Stability *in Vivo*

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ABSTRACT

Two new coupling agents were synthesized for making immunotoxins containing disulfide bonds with improved stability *in vivo*: sodium *S*-4-succinimidyloxycarbonyl-α-methyl benzyl thiosulfate (SMBT) and 4-succinimidyloxycarbonyl-α-methyl-α(2-pyridyldithio)toluene (SMPT). Both reagents generate the same hindered disulfide linkage in which a methyl group and a benzene ring are attached to the carbon atom adjacent to the disulfide bond and protect it from attack by thiolate anions.

An immunotoxin consisting of monoclonal anti-Thy-1.1 antibody (OX7) linked by means of the SMPT reagent to chemically deglycosylated ricin A-chain had better stability *in vivo* than an immunotoxin prepared with 2-iminothiolane hydrochloride (2IT) which generates an unhindered disulfide linkage. About 48 h after i.v. injection into mice, one-half of the SMPT-linked immunotoxin present in the blood was in intact form and one-half as released free antibody, whereas equivalent breakdown of the 2IT-linked immunotoxin was seen at about 8 h after injection. Consequently, the blood levels of the SMPT-linked immunotoxin remained higher than those of the 2IT-linked immunotoxin despite loss of immunotoxin from the blood by other mechanisms. Forty-eight h after injection, 10% of the injected dose of the SMPT-linked immunotoxin remained in the bloodstream as compared with only 1.5% of the 2IT-linked immunotoxin.

The ability of immunotoxins prepared with the new reagents to inhibit protein synthesis by Thy-1.1-expressing AKR-A/2 lymphoma cells *in vitro* was identical to that of immunotoxins prepared with 2IT or *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Clonogenic assays showed that fewer than 0.01% of AKR-A/2 cells survived exposure to high concentrations of OX7-abrin A-chain immunotoxins prepared with SMBT, 2IT, or SPDP. Twelve clones of cells which had survived treatment with the SMBT-linked immunotoxin were isolated. None of the clones was selectively resistant to the SMBT-linked immunotoxin when retested in cytotoxicity assays.

In conclusion, immunotoxins prepared with the new coupling agents should have improved antitumor activity *in vivo* because they are longer lived and do not break down so readily to release free antibody which could compete for the target antigens.

INTRODUCTION

Novel antitumor agents called "immunotoxins" have been synthesized in several laboratories by covalently linking the A-chain of ricin and other toxins to antibodies against tumor-associated antigens (reviewed in Refs. 1–5). These reagents bind to antigens on the target cell surface, are endocytosed, and the A-chain then traverses the membrane, probably of the endocytic vesicle, and kills the cell by inactivating its ribosomes.

Conjugation of the antibody and A-chain is generally accomplished by means of cross-linking agents that introduce a disulfide bond between the two proteins. Immunotoxins prepared with nonreducible linkages are consistently less cytotoxic than their disulfide-bonded counterparts indicating that reductive cleavage of the disulfide bond to release the A-chain in the cytosol may be an important step in the cytotoxic process (6, 7). The two disulfide coupling agents used in most laboratories are SPDP[2] (8) and 2IT (9). These agents are simple to use and give consistent products that are stable in *in vitro* systems and long-term storage.

The disulfide bond formed by the SPDP and 2IT reagents appears to be unstable *in vivo*. We have shown that, after injection into mice, immunotoxins prepared with these reagents break down with a half-life of about 8 h to release free antibody. This is true of immunotoxins prepared from the A-chains of abrin (10), native ricin (11) and ricin which has been chemically deglycosylated to prevent its clearance by the carbohydrate-recognition systems of the liver (11). In other laboratories, evidence has been found which supports (12–14) or opposes lability (15, 16). In those studies in which lability was not observed, the immunotoxins were prepared from native ricin A-chain and were cleared from the bloodstream so rapidly that the slower event of immunotoxin breakdown may not have been evident.

Breakdown of the linkage in the immunotoxin is a problem for two reasons: (*a*) there is less intact immunotoxin available to locate and kill the tumor cells; (*b*) the released antibody can compete with the immunotoxin for the target antigens (17) and, being much longer-lived (11), has greater opportunity to bind to them. The effectiveness of further injections of immunotoxin could therefore be diminished because the tumor cell antigens are masked by the released antibody from the first immunotoxin injection.

In the present study we synthesized two new coupling agents, SMBT and SMPT. These reagents were then used to prepare immunotoxins containing the same hindered disulfide linkage in which a methyl group and a benzene ring protect the disulfide bond from attack by thiolate anions. Immunotoxins prepared with the new reagents have improved *in vivo* stability and their toxicity to target cells is practically identical to that of immunotoxins prepared with SPDP or 2IT. A similar coupling agent, SPDB, with greater resistance to reduction was recently described by Worrell *et al.* (18). *In vivo* stability and cytotoxicity data for SPDB-linked immunotoxins have not yet been reported.

MATERIALS AND METHODS

Materials

Seeds of *Abrus precatorius* were kindly provided by Dr. S. Olsnes (Norsk Hydro's Institute for Cancer Research, Oslo, Norway). The seeds were of Indian origin. Crushed castor beans (*Ricinus communis*) were a gift from Croda Premier Oils, Ltd., Hull, England. The beans were from Central Africa.

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[2] The abbreviations used are: SPDP, *N*-succinimidyl-3-(2-pyridyldithio)-propionate; SMBT, sodium *S*-4-succinimidyloxycarbonyl-α-methyl benzyl thiosulfate; SMPT, 4-succinimidyloxycarbonyl-α-methyl-α(2-pyridyldithio)toluene; SBT, sodium *S*-4-succinimidyloxycarbonyl benzyl thiosulfate; 2IT, 2-iminothiolane hydrochloride; SPDB, *N*-succinimidyl-3-(2 pyridyldithio)butyrate; DTT, dithiothreitol; dg.ricA, deglycosylated ricin A-chain; abrA, abrin A-chain; IC_{50}, concentration that reduced [^{3}H]leucine incorporation by 50%; SDS, sodium dodecyl sulfate; OX7, monoclonal antibody directed against Thy-1.1; R10, monoclonal antibody directed against human glycophorin; GSH, reduced glutathione.

The hybridoma cell line, MRC OX7, secreting a mouse IgG1 subclass antibody to the Thy-1.1 antigen, was kindly provided by Dr. A. F. Williams (MRC Cellular Immunology Unit, University of Oxford). Details of its derivation have been published by Mason and Williams (19). The hybridoma cell line, LICR-LON-R10, secreting a mouse IgG1 subclass antibody to human glycophorin was kindly supplied by Dr. P. A. W. Edwards (Ludwig Institute, Sutton, England).

The Thy-1.1-expressing AKR-A lymphoma cell line was obtained from Professor I. MacLennan (Department of Experimental Pathology, Birmingham University, Birmingham, England). It was recloned to remove a mutant subpopulation which was resistant to immunotoxins prepared using the SPDP reagent but sensitive to immunotoxins prepared using the 2IT reagent (10). The recloned line is designated AKR-A/2.

Tissue culture medium RPMI 1640 and fetal calf serum were purchased from Gibco-Biocult, Ltd. (Paisley, Scotland). Agarose (Sea Plaque) was from FMC Corporation (Rockland, ME). Microplates with 96 flat-bottomed wells and tissue culture plates with 24 flat-bottomed wells were purchased from Flow Laboratories (Irvine, Scotland).

Sodium [^{125}I]iodide (IMS 30) and L-[4,5-^{3}H]leucine (TRK 170) were obtained from Amersham International (Amersham, England). The Iodo-Gen reagent for protein iodination was from Pierce (United Kingdom) Ltd. (Chester, England).

Chromatography media were Sephacryl S-200, Sepharose 4B, Sephadex G25 (fine grade), and Blue Sepharose CL-6B from Pharmacia Ltd. (Milton Keynes, England).

SPDP was purchased from Pharmacia, Ltd., and 2IT from Sigma, Ltd. (Poole, England). Thin layer chromatography (SiO_2) plates were from Merck (Kieselgel 60F). All other reagents were of analytical grade.

Synthesis of SBT

Bromotoluic acid (5.29 g; 25 mmol) was suspended in dioxan (10 ml) and was mixed with a solution of sodium thiosulfate (6.45 g; 26 mmol) in water (6 ml). The mixture was stirred at 40°C for 3 h during which time the solid dissolved and the thiosulfate derivative of toluic acid then crystallized out. The crystals (m.p. approximately 255°C with decomposition) were washed with cold water and dried under vacuum at 45°C to constant weight (3.90 g; 14 mmol; 55%). The solid was dissolved in dry dimethylformamide (5 ml) and mixed with a solution of *N*-hydroxysuccinimide (1.82 g; 16 mmol) and dicyclohexylcarbodiimide (2.97 g; 14 mmol) each in dry dimethylformamide (5 ml). The mixture was stirred for 16 h at room temperature and the urea was removed by filtration. The solvent was removed from the filtrate by rotary evaporation at 40°C using an oil pump and the *N*-succinimidyl derivative was recrystallized from methanol/$CHCl_3$. The yield of the white crystals was 3.47 g (66%). The overall yield for the synthesis was 36%. Melting point determinations showed decomposition at 120°C. The analysis

Requires: C 37.14, H 3.36, N 3.62, S 16.60, Na 5.95
Found: C 36.63, H 2.78, N 3.33, S 16.27, Na 6.01

was consistent with the structure shown in Fig. 1 ($C_{13}H_4NO_8S_2Na$).

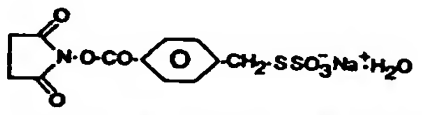

Fig. 1. The SBT reagent (formula weight, 385.4).

Synthesis of SMBT

p-Ethylbenzoic acid (5.15 g; 34 mmol) was dissolved in CH_2Cl_2 (45 ml) and solid *N*-bromosuccinimide (6.81 g; 38 mmol) was added followed by benzoylperoxide (0.08 g; 0.34 mmol) in CH_2Cl_2 (1 ml). The mixture was refluxed for 24 h. The white solid that remained was redissolved by the addition of further CH_2Cl_2 (40 ml) to the reaction mixture and the solution was extracted twice with water to remove succinimide. The CH_2Cl_2 solution was dried with anhydrous sodium sulfate and the solvent removed by rotary evaporation under reduced pressure. The white solid residue, α-bromoethylbenzoic acid, was recrystallized from isopropyl alcohol to give white crystals (m.p. 140°C) in good yield (5.62 g; 25 mmol; 72%). A solution of α-bromoethylbenzoic acid (0.60 g; 2.6 mmol) in dioxan (6 ml) was mixed with a solution of sodium thiosulfate (0.65 g; 2.6 mmol) in water (6 ml). The mixture was stirred for 16 h at room temperature and the solvents were removed in a vacuum at 40°C. The solid was washed with $CHCl_3$ and the thiosulfate derivative was recrystallized from water. The white crystals (shrinkage at 143°C; decomposition at 200°C) were recovered in poor yield (0.20 g; 0.72 mmol; 25%). The crystals were thoroughly dried and dissolved in dry dimethylformamide. The solution was mixed with solutions of dicyclohexylcarbodiimide (0.148 g; 0.72 mmol) and *N*-hydroxysuccinimide (0.090 g; 0.78 mmol) each in dry dimethylformamide (0.4 ml). The urea that had crystallized out after leaving the solution at room temperature for 16 h was removed by filtration. The solvent was removed from the filtrate by rotary evaporation at room temperature using an oil pump. The oily residue was redissolved in methyl ethyl ketone and undissolved solid was removed by filtration. The solvent was removed from the filtrate by rotary evaporation under reduced pressure and $CHCl_3$ was added to the residue which precipitated the product as a white solid that was dried under vacuum (0.17 g; 0.43 mmol; 72%; m.p. approximately 121°C with decomposition). The overall yield for the synthesis was 13%. The analysis

Requires: C 39.09, H 3.54, N 3.51, S 16.06, Na 5.76
Found: C 38.88, H 3.68, N 3.46, S 15.69, Na 5.68

was consistent with the structure shown in Fig. 2 ($C_{12}H_{12}NO_8S_2Na$).

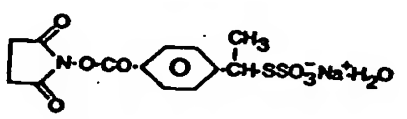

Fig. 2. The SMBT reagent (formula weight, 399.4).

Synthesis of SMPT

p-Ethylbenzoic acid was α-brominated and converted to the thiosulfate derivative as described in the preceding section. The thiosulfate (6.0 g; 20 mmol) was hydrolyzed by adding 5 N HCl (50 ml) and stirring at room temperature under nitrogen for 6 h. The reaction mixture was then extracted three times with ethyl acetate (50 ml) and dried over anhydrous sodium sulfate. The solvent was then removed in a vacuum to leave α-thioethylbenzoic acid (3.0 g; 16 mmol; 80%) as a white solid which was stored under nitrogen.

2-Pyridinesulfenylchloride was prepared by bubbling chlorine gas through a solution of 2,2-dipyridyldisulfide (2.3 g; 10 mmol) in dry dichloromethane (20 ml) for 30 min at room temperature. The solvent was then removed by rotary evaporation under reduced pressure and a solution of α-thioethylbenzoic acid (1.9 g; 10 mmol) in dry dioxan (10 ml) was added. The mixture was stirred vigorously overnight at room temperature under nitrogen. The yellow solid produced during the reaction was then partitioned between 0.05 M sodium phosphate buffer and ethyl acetate keeping the pH constant at 7.0. The organic layer was removed, dried over anhydrous sodium sulfate, and the solvent removed by rotary evaporation under reduced pressure to leave a yellow oil. Recrystallization from ethyl acetate/dichloromethane yielded colorless crystals (m.p. 130–132°C). The analysis

Requires: C 57.71, H 4.50, N 4.81, S 22.01
Found: C 57.92, H 4.63, N 4.89, S 21.99

was consistent with the product being 4-carboxy-α-methyl-α-(2-pyridyldithio)toluene ($C_{14}H_{13}NO_2S_2$).

To a solution of the 2-pyridyldithio derivative (1.9 g; 6.6 mmol) in dry dioxan (10 ml) were added dicyclohexylcarbodiimide (1.4 g; 6.8 mmol) and *N*-hydroxysuccinimide (0.79 g; 6.8 mmol) each dissolved in dry dioxan (approximately 4 ml). The mixture was stirred for 4 h at room temperature, filtered to remove the urea, and the solvent removed by rotary evaporation under reduced pressure. The product was purified by short column chromatography on Silica Gel H. Elution was effected with a gradient of CH_2Cl_2/ethyl acetate from 0 to 50% (v/v). On removal of the solvent, a colorless oil (0.6 g; 1.5 mmol; 24%) remained.

The product was homogeneous when analyzed by thin layer chromatography (SiO_2, ethyl acetate:CH_2Cl_2, 1:1) but attempts to crystallize it were unsuccessful. The overall yield for the synthesis was 2%. Nuclear magnetic resonance and infra-red analyses showed δH (d_4 methyl alcohol) 8.36 (1H, m, pyridyl); 7.96 (2H, m, phenyl); 7.73 to 7.53 (5H, m, phenyl and pyridyl); 7.06 (1H, m, pyridyl); 4.30 (1H, m, C<u>H</u>CH$_3$); 2.96 (4H, S, *N*-hydroxysuccinimide ester); and 1.76 (3H, m, C<u>H</u>C<u>H</u>$_3$) ν_{max} (CH_2Cl_2) 2920, 1770, 1740, and 1605 cm^{-1}. These analyses were consistent with the structure shown in Fig. 3 ($C_{16}H_{16}N_2O_4S_2$).

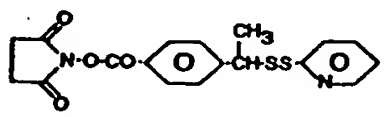

Fig. 3. The SMPT reagent (formula weight, 388.5).

Purification of Abrin A-Chain

Abrin was extracted from the seeds of *A. precatorius* by the method of Thorpe *et al.* (20). The toxin was split by reduction into its component chains and the A-chain was purified to homogeneity as described previously (21). The purified toxin and the A-chain (abrA) had median lethal dose values of 1.4 μg/kg and 12 mg/kg, respectively, when administered i.p. to adult BALB/c mice.

Deglycosylation and Purification of Ricin A-Chain

Ricin was purified from crushed castor beans by the method of Cumber *et al.* (21). A solution of the toxin (2.5 mg/ml) in 0.2 M sodium acetate buffer, pH 3.5, was treated for 1 h at 4°C with sodium metaperiodate and sodium cyanoborohydride at final concentrations of 10 and 20 mM, respectively, as described by Thorpe *et al.* (22). This procedure results in the destruction of approximately 50% of the mannose and most of the fucose residues present on the A-chain. The *N*-acetylglucosamine and most of the xylose residues are unaffected (23). The deglycosylated ricin A-chain was separated from the B-chain and was extensively purified by the method of Fulton *et al.* (24). The dg.ricA had a median lethal dose value of 15 mg/kg (as compared with 30 mg/kg for native ricA) when administered i.p. to adult BALB/c mice.

Purification of Antibodies

The monoclonal antibodies OX7 and R10 were purified from the blood and ascitic fluid of hybridoma-bearing BALB/c mice by the method of Mason and Williams (19).

Preparation of Immunotoxins

Buffer Solutions. Two buffer solutions were used during the synthesis of the immunotoxins: (*a*) 0.05 M sodium borate, pH 9.0, containing 1.7% (w/v) NaCl ("borate buffer"); (*b*) 0.01 M Na_2HPO_4-0.0018 M KH_2PO_4-0.17 M NaCl-0.0034 M KCl-0.001 M EDTA, pH 7.5 ("phosphate-EDTA buffer").

Derivatization of Antibody with SMBT. To a solution of antibody (20 mg) in borate buffer (4 ml) was added SMBT (216 μl; 1 mg/ml) in dry dimethylformamide. The final concentrations of SMBT and antibody were 0.13 and 0.032 mM, giving a 4-fold M excess of SMBT over antibody. The solution was stirred for 1 h at room temperature and a solution of DTT (40 μl; 15.4 mg/ml) in borate buffer was added, giving a final DTT concentration of 1 mM. The solution was stirred gently for a further 1 h at room temperature and a solution of 5,5′-dithio-bis(2-nitrobenzoic acid) [Ellman's reagent (25)] (40 μl; 87.2 mg/ml) in dimethylformamide was added, giving a final concentration of Ellman's reagent of 2.2 mM. The mixture was stirred gently for 1 h at room temperature and was applied to a column (20 x 1.6 cm) of Sephadex G25 (fine) equilibrated in nitrogen-flushed phosphate-EDTA buffer. The protein that eluted in the void volume of the column was concentrated to 10 mg/ml in an Amicon ultrafiltration cell fitted with a YM2 membrane. The average number of activated disulfide groups introduced into each antibody molecule was determined by reducing a sample of derivatized antibody solution with DTT and measuring the absorption of the released 3-carboxylato-4-nitrothiophenolate ion which has a molar absorptivity of 1.36×10^4 M^{-1} cm^{-1} at 412 nm (25). The number of activated disulfide groups introduced using the above conditions ranged between 1.5 and 1.8/molecule of antibody.

Derivatization of Antibody with SMPT. To a solution of antibody (20 mg) in borate buffer (2.67 ml) was added SMPT (267 μl; 0.48 mg/ml) in dry dimethylformamide. The final concentrations of SMPT and antibody were 0.11 and 0.045 mM, giving a 2.4-fold M excess of SMPT over antibody. The dimethylformamide was used at 10% v/v to keep the SMPT soluble. The solution was stirred for 1 h at room temperature and was applied to a column (30 x 1.6 cm) of Sephadex G25 (fine) equilibrated in nitrogen-flushed phosphate-EDTA buffer. The protein that eluted in the void volume of the column was concentrated to 10 mg/ml in an Amicon ultrafiltration cell fitted with a YM2 membrane. The average number of α-methyl-α-(2-pyridyldithio)toluoyl groups introduced into each antibody molecule was determined by reducing a sample of derivatized antibody solution with DTT and measuring the absorption of the released pyridine-2-thione which has a molar absorptivity of 8.08×10^3 M^{-1} cm^{-1} at 343 nm (8). The number of α-methyl-α(2-pyridyldithio)toluoyl disulfide groups introduced using the above conditions ranged between 1.5 and 2.0/molecule of antibody.

Coupling of SMBT- and SMPT-derivatized Antibodies to A-chain. A solution of abrA (10 mg) or dg.ricA (10 mg) in phosphate-EDTA buffer (7 ml) was treated for 30 min at room temperature with 50 mM DTT and applied to a column (30 x 2.2 cm) of Sephadex G25 equilibrated in nitrogen-flushed phosphate-EDTA buffer. The A-chain fraction (about 35 ml) that eluted from the column was added directly to the concentrated antibody solution (10 mg/ml; 2 ml) in the Amicon ultrafiltration cell giving a molar excess of A-chain over antibody of 2.5-fold. The mixture was then concentrated to about 10 ml and incubated at room temperature for 72 h under nitrogen. The mixture was removed from the ultrafiltration cell and treated with 0.2 mM cysteine for 6 h at room temperature to inactivate any activated disulfide groups remaining in the antibody component of the immunotoxin. These conditions do not cause splitting of immunotoxin. If this step were omitted, 20 to 30% of the M_r 180,000 immunotoxin interacted with plasma constituents in both *in vivo* and *in vitro* experiments to form a covalent adduct mainly of M_r 240,000. It is possible that residual activated disulfide groups on the antibody component react with the thiol group of albumin (M_r 67,000) to form the adduct.

Preparation of SPDP- and 2IT-linked Immunotoxins. The SPDP and 2IT coupling agents were used to link abrA or dg.ricA to OX7 antibody. Full details of the procedures have been published previously for both SPDP (6, 21) and 2IT (10, 26).

Purification of the Immunotoxins. The products of the conjugation reactions above were applied to a column (90 x 2.2 cm) of Sephacryl S-200 equilibrated in 0.05 M sodium phosphate buffer, pH 7.5, and eluted with the same buffer solution. The fractions of immunotoxin that eluted with a molecular weight of approximately 180,000 were pooled and fractionated on a Blue Sepharose column to remove free antibody and immunotoxin molecules containing more than one molecule of A-chain as described previously (27).

Analysis by polyacrylamide gel electrophoresis in SDS showed that the immunotoxins had an apparent molecular weight of 180,000 and that they contained one molecule of antibody linked to one molecule of A-chain. The concentration of immunotoxin was determined from absorbance measurements at 280 nm. IgG-ricA (M_r 180,000) has an $E^{0.1\%}_{1cm}$ at 280 nm of 1.29 assuming values of 1.40 for the antibody and 0.765 for the A-chain (28). IgG-abrA (M_r 180,000) has an $E^{0.1\%}_{1cm}$ at 280 nm of 1.30 assuming values of 1.40 for the antibody and 0.787 for the A-chain (28).

The immunotoxins have the structures shown in Fig. 4. The antibody components of the OX7 immunotoxins fully retained antigen-binding activity, as judged by fluorescence-activated cell sorter analyses on AKR-A/2 cells treated with antibody or immunotoxin at saturating and subsaturating concentrations. The A-chain components fully retained their ability to inhibit protein synthesis in reticulocyte lysates (29).

Rate Constants

Bovine IgG in borate buffer (5 mg/ml) was treated with SBT, SMBT, SPDP, or 2IT to introduce an average of approximately 5 molecules of

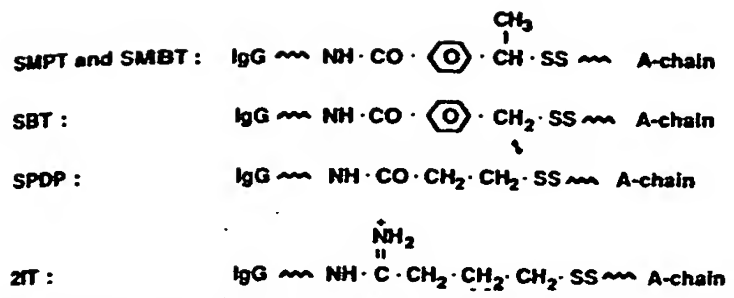

Fig. 4. Linkages formed by the different coupling agents.

coupling agent per molecule of protein. The derivatized IgG was treated with 1 mM DTT for 1 h followed by 2.2 mM Ellman's reagent for 1 h. This generated the same activated disulfide-leaving group (*i.e.*, 3-carboxylato-4-nitrothiophenolate ion) in all the derivatives. The derivatized IgG preparations were desalted on columns (20 x 1.6 cm) of Sephadex G25 equilibrated in 0.025 M sodium phosphate, pH 7.4, containing 0.15 M NaCl.

The IgG derivatives were treated with DTT (0.01 to 0.1 mM) or glutathione (0.1 to 1 mM) at 25°C and the rate of release of 3-carboxylato-4-nitrothiophenolate ion was measured at 412 nm using a Shimadzu (Model UV 240) spectrophotometer. DTT or glutathione was added at the same time to the reference cell which contained underivatized bovine IgG which had been treated with DTT followed by Ellman's reagent in the same way as the IgG derivatives. The second order rate constants were calculated from the equation

$$k = \frac{2.303}{t(a-b)} \log \frac{b(a-x)}{a(b-x)}$$

where a is the initial molar concentration of activated disulfide groups in the derivatized protein solution, b is the initial molar concentration of DTT or glutathione, and x is the molar concentration of released 3-carboxylato-4-nitrothiophenolate ion at time t s after adding DTT or glutathione.

Toxicity to AKR-A Cells in Tissue Culture

[^{3}H]Leucine Incorporation Assays. A suspension of AKR-A/2 cells was prepared at 10^5 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin (200 units/ml), and streptomycin (100 μg/ml) ("complete medium"). The cells were incubated for 24 h at 37°C with immunotoxins and other test materials using the microplate method described previously (29). [^{3}H]Leucine (1 μCi) was then added to each culture (200 μl) and the radioactivity that the cells incorporated was measured 24 h later.

Clonogenic Assays. A suspension of AKR-A/2 cells was prepared at 2×10^5 cells/ml in complete medium. The suspension was distributed in 50-ml volumes into 300-cm^2 tissue culture flasks and complete medium or medium containing immunotoxin (1 ml) was added to give a final immunotoxin concentration of 1.3×10^{-8} M. The cells were incubated for 24 h at 37°C in an atmosphere of 5% CO_2 in humidified air. The cells were then washed three times with complete medium.

Cells which had been incubated in medium alone were suspended at 120, 240, 360, 480, and 600 cells/ml in complete medium. Cells which had been treated with immunotoxin were suspended at a range of concentrations between 10^4 and 10^7 cells/ml in complete medium. A solution of 0.24% w/v agarose in complete medium at 45°C was dispensed in 1-ml volumes into Petri dishes (35-mm diameter) and cooled for 1 h at 4°C to solidify the agarose. To aliquots of cell suspension (0.5 ml) in sterile tubes at 4°C was added a solution of 0.24% w/v agarose in complete medium (2.5 ml) at 45°C. The suspensions were then mixed, transferred in 1-ml volumes to the agar-coated Petri dishes, and were cooled for 1 h at 4°C to solidify the agarose. The Petri dishes were then incubated at 37°C for 10 days and the number of colonies containing about 100 cells or more was counted using an inverted microscope. The percentage of cells that survived exposure to the immunotoxins was calculated by comparing their cloning efficiency to that of untreated cells for that particular experiment. The cloning efficiency of untreated cells ranged from 65 to 76%.

Stability and Blood Clearance Measurements

Measurements of the stability and blood clearance rates of the immunotoxins were performed as previously described (11). Briefly, the purified OX7-SMPT-dg.ricA and OX7-2IT-dg.ricA immunotoxins were radioiodinated with ^{125}I to a specific activity of approximately 10^7 cpm/μg. Groups of three adult male specific-pathogen-free BALB/c/ICRF mice were given injections i.v. of 10 μg of radioiodinated immunotoxins and samples of blood were drawn from the tail vein at various time intervals and transferred to heparin-coated tubes. The radioactivity in the blood samples (50 μl) was measured. The samples were centrifuged at 10,000 × g for 2 min and the plasma was removed. The radioactivity in 20 μl plasma was counted and the samples were stored in liquid N_2. At the end of the experiment, volumes of plasma samples containing 8000 cpm each were electrophoresed on 5 to 10% polyacrylamide gels (1 mm thick) containing 1% SDS.

Autoradiographs of the dried gels were scanned and the area under the immunotoxin (M_r 180,000) peak and the released antibody (M_r 150,000) peak was divided by the total area under all the peaks to determine the proportion of radioactivity in the plasma that corresponded to intact immunotoxin or released antibody. Calibration experiments had previously shown that the area under each peak was directly proportional to the cpm it contained. Analysis of the immunotoxin by SDS-polyacrylamide gel electrophoresis under reducing conditions showed that the specific activity of the released antibody was somewhat less (9.1×10^6 cpm/μg) than that of the intact immunotoxin (10×10^6 cpm/μg). Correction was therefore made for this difference when calculating the amount of released antibody in the bloodstream. Clearance measurements were expressed as a percentage of the injected dose assuming that the mice had a blood volume of 2.18 ml/25 g body weight (30).

A two-compartment open pharmacokinetic model was fitted to the plasma levels of immunotoxins and released antibody using a computerized nonlinear least-squares regression analysis (31). A weighting function of $1/(Y + \hat{Y})^2$ was applied to all data points (32). These analyses yielded the half-lives of the immunotoxins in the α and β phases of clearance. Also, the half-lives of splitting of the immunotoxins to free antibody and A-chain were calculated using an extension of the same model to be described in a subsequent report.[3]

RESULTS

Rates of Reduction of IgG Derivatized with Various Coupling Agents

Bovine IgG was reacted with SMBT, SBT, SPDP, or 2IT and then treated with DTT followed by Ellman's reagent to form antibody derivatives in which the same activated disulfide group was present in all. The antibody derivatives differed only in the groups through which the activated disulfide group was attached to the protein, as in Fig. 4.

The release of 3-carboxylato-4-nitrothiophenolate ion when the antibody derivatives were treated with DTT or glutathione followed approximately second order kinetics, although the rate constant was greatest during the initial phase of reduction (Fig. 5). The activated disulfide groups reduced first probably occupied positions on the protein that were accessible to the reducing agents, whereas the more resistant groups were probably buried more deeply within the protein. The second order rate constants listed in Table 1 have been calculated at the point at which 50% of the leaving groups have been released and define the relative ease of reduction of the disulfide bonds formed by the different reagents.

The stability of the different linkages depended upon the degree of steric hindrance afforded by the groups adjacent to

[3] D. C. Blakey, D. N. Skilleter, R. J. Price, H. Newell, and P. E. Thorpe, Comparison of the pharmacokinetics and hepatotoxic effects of saporin and ricin A-chain immunotoxins, manuscript in preparation.

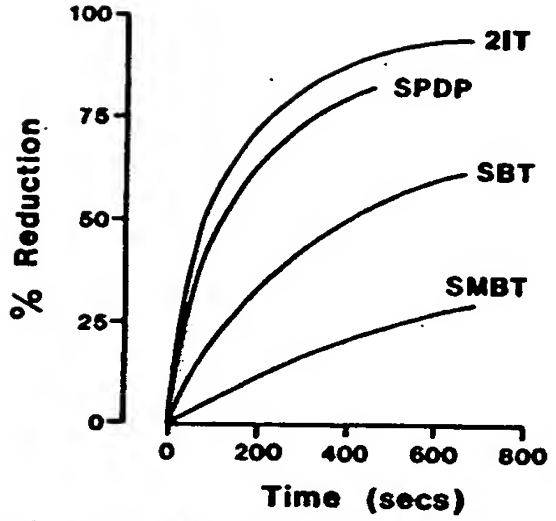

Fig. 5. Rates of reduction by DTT of IgG derivatized with various coupling agents. Bovine IgG was reacted with SMBT, SBT, 2IT, or SPDP and the derivatives were reduced with DTT and treated with Ellman's reagent to form antibody derivatives in which the same activated disulfide group was present in all. The derivatives were then treated with 0.03 mM DTT at pH 7.4 and 25°C. The rate of release of the 3-carboxylato-4-nitrothio phenolate group was followed spectrometrically at 412 nm. The A_{412} at various times after adding DTT is expressed as a percentage of the A_{412} after complete reduction with 5 mM DTT.

Table 1 *Rate constants for the reduction of IgG derivatized with various coupling agents by DTT or glutathione*

Bovine IgG was reacted with the above coupling agents and the derivatives were reduced with DTT and treated with an excess of Ellman's reagent to form antibody derivatives in which the same activated disulfide group was present in all. The derivatives were then treated with DTT (0.01 to 0.1 mM) or glutathione (0.1 to 1 mM) at pH 7.4 and 25°C. The rate of release of the 3-carboxylato-4-nitrothiophenolate group was measured spectrophotometrically as in Fig. 5. The second order rate constants shown below were calculated at the point at which 50% of the activated disulfide groups had been removed (see "Materials and Methods"). Repeated determinations gave results that did not differ by more than 15% from those shown.

Coupling agent	Rate constant (liters·mol^{-1}·s^{-1})	
	DTT	Glutathione
SMBT	14	2.5
SBT	72	Not done
SPDP	250	52
2IT	320	165

the disulfide bond. As shown in Fig. 5 and Table 1, the SMBT reagent gave the most stable linkage, probably because the disulfide bond was protected by the methyl group and, to a lesser extent, by the benzene ring. The next most stable linkage was given by SBT which has a benzene ring in the same position as in SMBT but which lacks the methyl group. The least stable linkages were given by the SPDP and 2IT reagents in which the disulfide bond is essentially unprotected. Thus, in summary, the ratios of the rate constants for the reduction of the SMBT, SBT, SPDP, and 2IT linkages with DTT were 0.04:0.23:0.78:1, respectively. The reciprocals of these ratios give the relative stability of the SMBT, SBT, SPDP, and 2IT linkages as 24:4.5:1.3:1, respectively. These differences were even more marked when glutathione was used as the reducing agent (see Table 1).

Cytotoxicity to AKR-A/2 Lymphoma Cells

[^{3}H]Leucine Incorporation Assays. OX7-SMPT-dg.ricA had identical ability to OX7-2IT-dg.ricA to reduce protein synthesis by Thy-1.1-expressing AKR-A/2 cells in tissue culture (Fig. 6). Both immunotoxins reduced the [^{3}H]leucine incorporated by the cells by 50% at a concentration (the IC_{50}) of 6×10^{-13} M. They were about 10-fold more potent even than ricin which had an IC_{50} of 8×10^{-12} M. The toxic effects were specific. Unconjugated OX7 was not toxic at 10^{-7} M and unconjugated dg.ricA and the control immunotoxin, R10-SMPT-dg.ricA, were only toxic at concentrations in excess of 10^{-8} M.

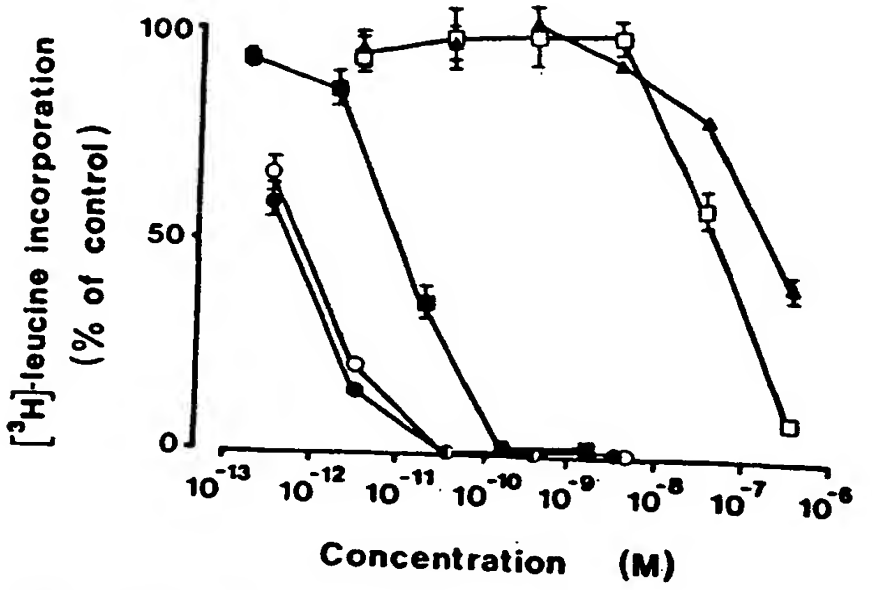

Fig. 6. Cytotoxic effects of OX7-SMPT-dg.ricA (○) and OX7-2IT-dg.ricA (●) upon AKR-A/2 lymphoma cells in tissue culture. The cells were incubated for 48 h with the immunotoxins or with R10-SMPT-dg.ricA (▲), dg.ricA (□), or ricin (■). *Points*, geometric means of triplicate measurements of [^{3}H]leucine incorporated by the cells during the final 24-h period of culture expressed as a percentage of the incorporation in untreated cultures. *Bars*, one SD about the mean unless smaller than the *points* as plotted. Mean [^{3}H]leucine incorporation in untreated cultures was 42,000 dpm.

Table 2 *Cytotoxic effects of OX7-abrA immunotoxins upon AKR-A/2 cells in vitro*

Immunotoxin	IC_{50} in [^{3}H]leucine uptake assays[a] (M)	Clonogenic assays[b] (% surviving cells)
OX7-SMBT-abrA	2.6×10^{-12}	0.0086 ± 0.0003
OX7-SPDP-abrA	2.0×10^{-12}	0.0049 ± 0.0021
OX7-2IT-abrA	3.0×10^{-12}	0.0037 ± 0.0002
OX7-abrA cocktail[c]	3.3×10^{-12}	0.0053 ± 0.0003
R10-SMBT-abrA	$>3 \times 10^{-8}$	90.0 ± 8.5
R10-SPDP-abrA	$>3 \times 10^{-8}$	73.5 ± 12.0
R10-2IT-abrA	$>3 \times 10^{-8}$	74.0 ± 1.4

[a] IC_{50} as determined in experiments such as is shown in Fig. 6.
[b] Immunotoxins were applied to the cells at 1.3×10^{-8} M. Results are the arithmetic means ± one SD of triplicate determinations. The plating efficiency of untreated cells was 76%.
[c] The cocktail contained SPDP-, 2IT- and SMBT-linked immunotoxin at 0.43×10^{-8} M each (total, 1.3×10^{-8} M).

In other experiments, OX7-abrA immunotoxins prepared with SMBT, SPDP, and 2IT were found to have very similar cytotoxicity to AKR-A/2 cells. Their IC_{50} values in [^{3}H]leucine incorporation assays ranged between 2.0 and 3.0×10^{-12} M (Table 2). Again, their toxic effects were specific. Control immunotoxins prepared from the R10 antibody were not toxic to AKR-A/2 cells at concentrations as high as 3×10^{-8} M, and none of the OX7-abrA immunotoxins was toxic to EL4 cells, a mouse lymphoma line which lacks Thy-1.1, at 3×10^{-8} M.

Clonogenic Assays

Clonogenic assays were used to quantify the survival of AKR-A/2 cells after treatment with the different OX7-abrA immunotoxins at the saturating concentration of 1.3×10^{-8} M. The 2IT- and SPDP-linked immunotoxins killed all but 0.0037 and 0.0049% of the cells, respectively, whereas the SMBT-linked immunotoxin killed all but 0.0086% of the cells (Table 2). This difference between the killing obtained with the SMBT-linked immunotoxin and that with the other two immunotoxins is statistically significant ($P < 0.05$) but is very small in view of the fact that more than 99.99% of the cells were killed with all three immunotoxins. A similar significant difference was obtained when the experiment was repeated.

Twelve clones of cells which survived exposure to OX7-SMBT-abrA were isolated and their sensitivity to the SMBT-,

2IT-, and SPDP-linked immunotoxins was tested in [^{3}H]leucine incorporation assays. Of 11 clones, one clone was fully resistant to all 3 immunotoxins, 4 were fully sensitive, and 6 showed intermediate sensitivity. None of these clones showed more than a 3-fold difference in their sensitivity to any individual immunotoxin indicating that the type of linkage used to form the immunotoxin does not usually affect the nature of the surviving cells (results not shown). In contrast, the 12th clone (S6A2) was 5- to 10-fold more sensitive to the 2IT-linked immunotoxin than it was to the other two immunotoxins. The IC_{50} values for this clone were 1×10^{-9} M with the 2IT-linked immunotoxin and 5×10^{-9} and 1×10^{-8} M with the SPDP- and SMPT-linked immunotoxins, respectively. Importantly, none of the clones studied was resistant only to the SMBT-linked immunotoxin suggesting that an inability to split the hindered disulfide bond in the SMBT linkage was not a cause of mutant cell survival.

Stability and Clearance Rate of Immunotoxins *in Vivo*

Fig. 7 shows autoradiographs of SDS gels of blood samples from mice given injections of radioiodinated OX7-SMPT-dg.ricA or OX7-2IT-dg.ricA at various earlier time intervals. The immunotoxin preparations that were injected contained a single major component (M_r 180,000) consisting of one molecule of antibody and one molecule of A-chain. After injection, both immunotoxins broke down to give a long-lived product (M_r 150,000) corresponding to free antibody. At later time points (Fig. 7, *lanes 9* and *10*) a minor component (M_r 210,000) was also seen on the gels. Free A-chain (M_r 30,000) was not seen at any time point probably because it was very rapidly cleared (33).

The rate at which OX7-SMPT-dg.ricA broke down to release free antibody *in vivo* was slower than that of OX7-2IT-dg.ricA. Plasma samples contained approximately equal amounts of intact immunotoxin and released antibody 48 h after injection of OX7-SMPT-dg.ricA (Fig. 7*a*, *lane 8*) as compared with after about 8 h in recipients of OX7-2IT-dg.ricA (Fig. 7*b*, *lane 6*). The released antibody had a specific activity of 9.1×10^6 cpm/μg which is lower than that of the intact immunotoxin (10×10^6 cpm/μg) so that the true amount of released antibody is slightly higher than appears to be present on the gels. The rate of splitting of OX7-2IT-dg.ricA was very similar to that of OX7-2IT-abrA and OX7-SPDP-abrA which, as we previously reported (10), have the same stability.

The breakdown products of the immunotoxins were characterized by absorbing the plasma samples with anti-ricin antibody coupled to Sepharose and rerunning the gels. This procedure entirely removed the intact immunotoxin (M_r 180,000) leaving behind the M_r 150,000 component corresponding to released antibody and the M_r 210,000 component (results not shown). Absorption of the plasma samples with Sepharose coupled to an antibody of irrelevant specificity removed none of the radioiodinated components, confirming that the absorption of immunotoxin by antiricin coupled to Sepharose was antigen specific. The M_r 210,000 component therefore lacked ricin A-chain and probably arose by displacement of the A-chain from the immunotoxin by a serum component with a calculated molecular weight of approximately 60,000. One possibility is that this serum component is albumin (M_r 67,000) which, having a free thiol group, could potentially displace the A-chain and remain attached to the antibody.

In Fig. 8 are shown the amounts of OX7-SMPT-dg.ricA and OX7-2IT-dg.ricA remaining in intact (M_r 180,000) form in the blood plasma of mice at various times after injection. The clearance curves were biphasic, having an initial rapid α-phase followed by a slower β-phase. A computerized analysis of the clearance data using an open two-compartment pharmacokinetic model gave α-phase half-lives of 1.2 ± 0.2 (SE) h for OX7-SMPT-dg.ricA and 2.3 ± 0.3 h for OX7-2IT-dg.ricA. In the β-phase, the half-lives were 22 ± 1 h for OX7-SMPT-dg.ricA and 11 ± 1 h for OX7-2IT-dg.ricA. The α- and β-phase half-lives for native OX7 antibody were 6.7 ± 1.2 h and 118 h, respectively. Using a computerized analysis to be described elsewhere,[3] the OX7-SMPT-dg.ricA was calculated to split up to give free antibody with a half-life of 21.8 h as compared with 6.5 h for OX7-2IT-dg.ricA.

As a consequence of its better stability, 10% of the injected dose of OX7-SMPT-dg.ricA remained intact in the blood plasma 48 h after injection as compared with 1.5% of OX7-2IT-dg.ricA and 35% of native OX7 antibody (Fig. 8).

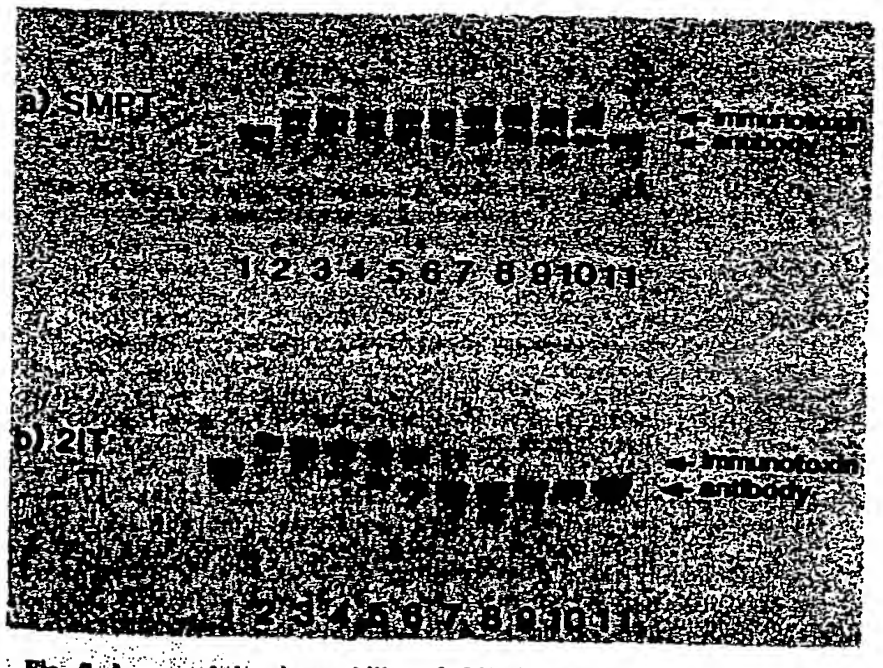

Fig. 7. Improved *in vivo* stability of OX7-dg.ricA prepared with SMPT. Autoradiographs of SDS-polyacrylamide (5 to 10%) gels of plasma samples taken at various time intervals from mice which had been given i.v. injections of (*a*) OX7-SMPT-dg.ricA or (*b*) OX7-2IT-dg.ricA. *a* and *b*, *lanes 1* and *11*, radioiodinated native OX7 antibody (M_r 150,000); *lane 2*, immunotoxin (mainly M_r 180,000) before injection. *a*, *lanes 3* to *10*, plasma samples taken 0.15, 2, 4, 8, 24, 48, 72, and 96 h after injection, respectively. *b*, *lanes 3* to *10*, plasma samples taken 0.15, 2, 4, 8, 24, 48, 72, and 120 h after injection, respectively.

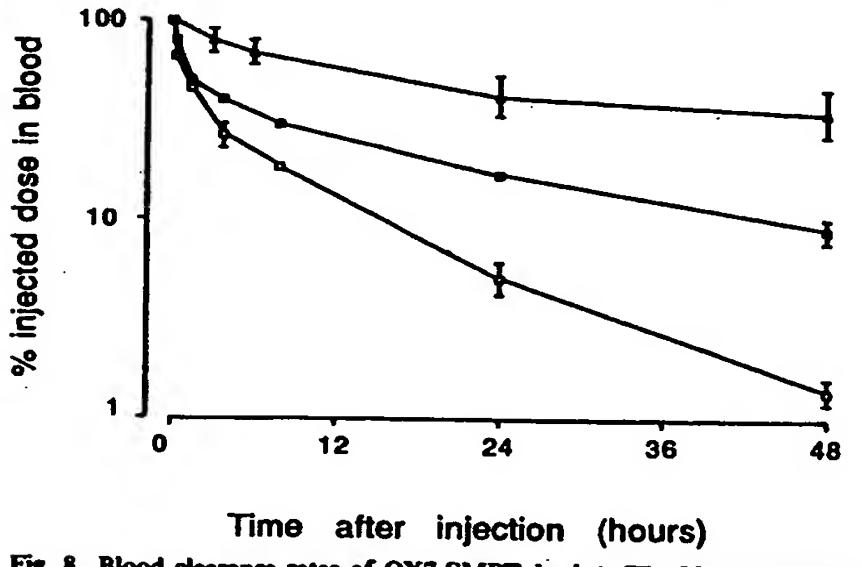

Fig. 8. Blood clearance rates of OX7-SMPT-dg.ricA (■), OX7-2IT-dg.ricA (□), and OX7 (Δ). Mice were given i.v. injections of radioiodinated immunotoxins and blood samples were removed at various time intervals later. The percentage of the injected dose that corresponded to intact (M_r 180,000) immunotoxin was determined by scanning autoradiographs of SDS gels such as those in Fig. 7. *Points*, geometric mean and SD (*bars*) of results obtained in three mice. OX7 derivatized with 2IT or SMPT followed by reduction and alkylation had identical blood clearance rates to native OX7 antibody (results not shown).

In contrast with the *in vivo* results, breakdown of the immunotoxins was not seen when they were incubated at 37°C for 24 h in 2 ml of mouse plasma or heparinized whole blood.

DISCUSSION

In the present study, we synthesized two new coupling agents, SMBT and SMPT, for preparing immunotoxins containing disulfide bonds with improved stability *in vivo*.

The SMBT and SMPT reagents generate the same protected disulfide linkage in which a methyl group and a benzene ring are attached to the carbon atom adjacent to the disulfide bond. However, the SMPT reagent is to be preferred for forming immunotoxins for two reasons: (*a*) it is simpler to use because it introduces the activated disulfide group in a single step, whereas the SMBT reagent introduces a thiosulfate group which has to be reduced and the resultant thiol activated with Ellman's reagent; (*b*) the SMPT coupling procedure does not involve exposing the antibody to DTT which potentially could cleave interchain disulfide bonds in the antibody and risk light chain loss.

In model experiments with antibody derivatized with different coupling agents, the protected disulfide bond formed by the SMBT reagent was found to have 24-fold greater resistance to reduction with DTT than the unprotected disulfide bonds formed by the SPDP and 2IT reagents. Both the benzene ring and the α-methyl group contribute to the greater stability of the SMBT linkage. This is indicated by our finding that antibody derivatized with a further coupling agent, SBT, which gives a linkage having a benzene ring in the same position as in the SMBT linkage but which lacks the α-methyl group, was about four times more resistant to thiol attack than the SPDP and 2IT linkages (see Table 1).

A similar coupling agent, SPDB, was recently synthesized by Worrell *et al.* (18). The SPDB reagent also generates a linkage with a methyl group substituted on the carbon atom adjacent to the disulfide bond but it has a smaller —$CH_2 \cdot CH_2$— group in place of the benzene ring in the SMPT linkage and so may have inferior stability.

A dg.ricA immunotoxin prepared with SMPT broke down more slowly *in vivo* to release free antibody than an immunotoxin containing the unhindered 2IT linkage. Consequently, the β-phase half-life of the SMPT-linked immunotoxin was increased to 22 h as compared with 11 h for the immunotoxin prepared with the unhindered disulfide bond. In the study by Worrell *et al.* (18) increased stability of the SPDB linkage was not directly demonstrated but was inferred from the fact that the β-phase half-life of the SPDB-linked immunotoxin was increased to 14 h as compared with 10 h for an immunotoxin prepared with an unhindered linkage.

The mechanism by which immunotoxins are broken down *in vivo* is unknown. It has been suggested from the finding that immunotoxins do not break down significantly when incubated in mouse plasma or whole blood *in vitro*, that the splitting must occur within one of the organs or tissues of the animal, possibly the liver (11). However, this is not necessarily true, because GSH, the major free plasma thiol, is very rapidly lost from plasma *in vitro* by mechanisms that do not appear to involve oxidation by molecular oxygen (34). *In vivo*, GSH is continually being manufactured by the liver and is maintained in plasma at a level of about 24 μM. Thus it is possible that the disulfide bond in immunotoxins is slowly split by GSH in the blood *in vivo* and that placing hindering groups around the disulfide bond protects it from attack. Alternatively, the splitting could be due to the action of a disulfide reductase and the hindering groups frustrate enzymatic attack.

OX7-abrA and OX7-dg.ricA prepared with the SMBT or SMPT reagents had identical ability to inhibit [^{3}H]leucine incorporation by AKR-A/2 cells to immunotoxins prepared with SPDP or 2IT. We have since obtained similar results in other *in vitro* test systems using immunotoxins constructed from antibodies with a variety of different specificities. Thus strengthening the linkage in the immunotoxin does not weaken their cytotoxic activity as measured by gross reductions in protein synthesis 24 to 48 h after adding the immunotoxin to the cells.

Clonogenic assays revealed that 0.009% of AKR-A/2 cells survived exposure to high concentrations of OX7-SMBT-abrA whereas the survival after treatment with OX7-SPDP-abrA and OX7-2IT-abrA was 0.005 and 0.004%, respectively. This difference was statistically significant and prompted us to examine the immunotoxin sensitivity of 12 clones of cells that had survived exposure to the SMBT-linked immunotoxin. None of the clones was selectively resistant to the SMBT-linked immunotoxin when retested with the immunotoxins showing that an inability to split the hindered disulfide linkage and release the A-chain within the cytosol was not a cause of mutant cell survival, or, if it were, it was not a stable mutation.

In conclusion, immunotoxins prepared using the SMBT and SMPT reagents should have superior antitumor activity *in vivo* because, being more stable, they have more time to locate and kill the tumor cells and they release less free antibody which can compete for the target antigens.

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